

In another embodiment, the present invention provides a method for isolating and or purifying a uricase from a solution of uricase containing, for example, cellular and subcellular debris from, for example, a recombinant production process. Preferably, the method of purification takes advantage of the limited solubility of mammalian uricase at low pH (Conley et al. 1979), by washing the crude recombinant extract at a pH of about 7 to about 8.5 to remove a majority of the proteins that are soluble at this low pH range, whereafter active uricase is solubilized in a buffer, preferably sodium carbonate buffer, at a pH of about 10-11, preferably about 10.2. The solubilized active uricase may then be applied to an anion exchange column, such as a Q Sepharose column, which is washed with low to high salt gradient in a buffer at a pH of about 8.5, after which purified uricase is obtained by eluting with a sodium chloride gradient in sodium carbonate buffer at a pH of about 10 to about 11, preferably about 10.2. The enzyme may be further purified by gel filtration chromatography at a pH of about 10 to about 11. At this stage, the enzyme may be further purified by lowering the pH to about 8.5 or less to selectively precipitate uricase, but not more soluble contaminants. After washing at low pH (7-8) the uricase is then solubilized at a pH of about 10.2. The uricase preparation could then be analyzed by methods known in the art of pharmaceutical preparation, such as, for example, any one of high performance liquid chromatography (HPLC), other chromatographic methods, light scattering, centrifugation and/or gel electrophoresis.

Brief Description of the Drawings

Figure 1. SDS-mercaptoethanol PAGE (12% gel) analysis

Figure 2. Circulating life of native and PEGylated PBC uricase.

Figure 3. Relationship of serum uricase activity to the serum and urine concentrations of uric acid.

Figure 4. Maintenance of circulating level of uricase activity (measured in serum) after repeated injection.

Figure 5 shows the deduced amino acid sequences of pig-baboon chimeric uricase (PBC uricase) and porcine uricase containing the mutations R291K and T301S (PKS uricase), compared with the porcine and baboon sequences.

Figure 6. Comparison of amino acid sequences PKS and pig uricase.

Figure 7. Comparison of amino acid sequences of PBC and PKS.

Figure 8. Comparison of amino acid sequences of PBC and pig uricase.

Figure 9. Comparison of amino acid sequence of pig uricase and D3H.

5 Figure 10. Comparison of amino acid sequences of PBC and D3H.

Figure 11-1 and 11-2. Bestfit (GCG software) comparison of coding sequences of the cDNAs of PKS and pig uricase.

Figure 12-1 and 12-2. Bestfit (GCG software) comparison of coding sequences of the cDNAs of PKS and baboon uricase.

10 Figure 13-1 and 13-2. Bestfit (GCG software) comparison of coding sequences of the cDNAs of PBC and pig uricase.

Figure 14-1 and 14-2. Bestfit (GCG software) comparison of coding sequences of the cDNAs of PBC and baboon uricase.

15 Detailed Description of the Invention

The present invention provides uricase proteins which are useful intermediates for improved uricase conjugates of water-soluble polymers, preferably poly(ethylene glycols) or poly(ethylene oxides), with uricases. Uricase, as used herein, includes
20 individual subunits as well as the native tetramer, unless otherwise indicated.

Although humans do not make an active enzyme, uricase mRNA transcripts have been amplified from human liver RNA (Wu et al. 1992). It is theoretically possible that some human uricase transcripts are translated; even if the peptide products were not full length or were unstable, they could be processed by antigen presenting cells and
25 play a role in determining the immunologic response to an exogenous uricase used for treatment. It may, in theory, be possible to reconstruct and express a human uricase cDNA by eliminating the two known nonsense mutations. However, in the absence of selective pressure, it is very likely that deleterious missense mutations have accumulated in the human gene during the millions of years since the first nonsense mutation was
30 introduced (Wu et al. 1989; Wu et al. 1992). Identifying and "correcting" all mutations to obtain maximal catalytic activity and protein stability would be very difficult.

The present inventors have appreciated that there is a high degree of homology (similarity) between the deduced amino acid sequence of human uricase to those of pig (about 86%) and baboon (about 92%) (see, Figures 6-14, for example of measure of similarity), whereas homology (similarity) between human and *A. flavus* uricase is <40% (Lee et al. 1988; Reddy et al. 1988; Wu et al. 1989; Legoux et al. 1992; Wu et al. 1992). The present invention provides recombinantly produced chimeric uricase proteins from two different mammals which have been designed to be less immunoreactive to humans than more distantly related fungal or bacterial enzyme. Use of a mammalian uricase derivative is expected to be more acceptable to patients and their physicians.

Experience has shown that activated PEGs such as have been used to make PEG-ADA and to modify other proteins attach via primary amino groups of the amino terminal residue (when present and unblocked) and epsilon-amino groups of lysines. This strategy is useful both because mild reaction conditions can be used, and because positively charged lysines tend to be located on the surfaces of proteins. The latter is important since for any therapeutic protein the desired effects of PEGylation will depend in part on the characteristics of the PEG polymer (e.g. mass, branched or unbranched structure, etc.) as well as on the number and distribution of PEG attachment sites of the protein relative to the epitopes and structural elements that determine function and clearance of the protein. A strategy for enhancing the ability of PEGylation to 'mask' epitopes and reduce immunogenicity by semi-selectively introducing novel lysine residues for potential PEG addition has been devised (Hershfield et al. 1991). This strategy employs mutagenesis to replace selected arginine codons with lysine codons, a substitution that maintains positive charge and has minimal effect on computer-predicted indices of surface probability and antigenicity (useful when only amino acid sequence is known).

As an experimental test of this strategy, recombinant *E. coli* purine nucleoside phosphorylase (EPNP) (Hershfield et al. 1991) has been used. Arg-to-Lys substitutions at 3 sites were introduced, increasing the number of lysines per subunit from 14 to 17, without altering catalytic activity. The purified triple-mutant retained full activity after modification of ~70% of accessible NH₂ groups with excess disuccinyl-PEG5000.